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CHOLESTEROL EFFECT ON ENZYME ACTIVITY OF THE SARCOLEMMAL ($Ca^{2+} + Mg^{2+}$)-ATPase FROM CARDIAC MUSCLE

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(1) The effect of cholesterol incorporation and depletion of the cardiac sarcolemmal sacs on ($Ca^{2+} + Mg^{2+}$)-ATPase activity was examined. (2) Cholesterol incorporation to the sarcolemmal sacs was achieved utilizing an in vivo and an in vitro procedure. Cholesterol depleted membranes were obtained in vitro after incubation of the sarcolemmal sacs with inactivated plasma. (3) Arrhenius plots of the ($Ca^{2+} + Mg^{2+}$)-ATPase activity showed a triphasic curve when the assays were carried out using a temperature range between 0 and 40°C. (4) The sarcolemmal ($Ca^{2+} + Mg^{2+}$)-ATPase activity was shown to be inversely proportional to the cholesterol concentration of the membranes, showing a low ATPase activity with a high cholesterol content and a high ATPase activity when the cholesterol concentration was low. (5) Although the ($Ca^{2+} + Mg^{2+}$)-ATPase activity was found to be inhibited in the cholesterol incorporated sarcolemmal sacs, the withdrawal of small amounts of cholesterol from the membranes produced an important stimulatory effect. (6) Changes in ($Ca^{2+} + Mg^{2+}$)-ATPase activity due to variation in the membrane cholesterol concentration were shown to be reversible. (7) Our results indicate the possibility of a slow exchange of cholesterol between the tightly bound lipid surrounding the ($Ca^{2+} + Mg^{2+}$)-ATPase and the bulk lipid of the sarcolemma.

Introduction

Nowadays, there is considerable evidence that many functions carried out by biological membranes are influenced or controlled by the composition and physical state of the lipids that constitute them [1–3]. Among these lipids, continuous attention over the years has been given to the effect of cholesterol on biological membranes [4–5]. Although cholesterol is a major constituent of most biological membranes, many of its functions are still not well understood. However, there is a general agreement concerning the effect of cholesterol upon the lipid motional freedom or membrane 'fluidity' [6].

Cholesterol and sterols in general exert either a condensing or a liquifying effect depending on the physical state of these membranes. When the membrane lipids are arranged in a liquid-crystalline state, cholesterol decreases chain mobility, in contrast, when these lipid are arranged in a gel state, cholesterol increases the mobility. Therefore, one or the main functions of cholesterol in biological membranes seems to be to modulation of the molecular motion of phospholipids towards an intermediate state [6].

Although there has been throughout the years some confusion concerning the effect of cholesterol upon different enzyme activities, nowadays it is known that changes in lipid structure induced by

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lowering or increasing the temperature, alter the kinetics of enzymes associated with different membranes directly affecting their physiological function [7–11]. Since cholesterol has been reported to affect the enzyme activity of different membrane located ATPases [12–14], the following study has been undertaken in order to determine to which extent cholesterol might affect the $(Ca^{2+} + Mg^{2+})$-ATPase located in the sarcolemma of the cardiac muscle cells.

Materials and Methods

Incorporation of cholesterol to the sarcolemma in vivo. The cholesterol content of the sarcolemmal membranes was altered in vivo by the addition of 2% cholesterol to the diet of New Zealand White rabbits with approximate weights between one and two kilograms during four to five weeks. Their cholesterol levels in plasma were determined every week by means of a colorimetric determination employing an enzymatic system based in the oxidation of plasma cholesterol (Boehringer Mannheim). After three weeks with the special diet, when the plasma cholesterol level had substantially increased, the sarcolemmal sacs were isolated from the ventricular muscle as reported elsewhere [15,16] and the cholesterol content of the different samples measured utilizing the same enzymatic method employed for the determination of plasma cholesterol.

Incorporation and depletion of cholesterol from the sarcolemma in vitro. In vitro changes of the sarcolemmal cholesterol content were accomplished by incubating the sacs from control animals in treated human plasma supplemented with cholesterol at 37 °C for different times, based on the method described by Shinitzky [17]. The sarcolemmal sacs were incubated in a medium containing 15 mg of cholesterol previously dissolved in 1 ml of 2% dimethyl sulfoxide and diluted 1 : 100 times in 10% (v/v) serum. The serum containing fresh human plasma was previously incubated at 57 °C for 30 min and diluted to 10% with a buffer containing 55 mM NaH_2PO_4 (pH 7.1). After this treatment, the sacs were spun down three times at $7000 \times g$ for 10 min and washed with the same phosphate buffer.

The extraction of the native cholesterol from the sarcolemmal sacs was carried out also according to Shinitzky [17]. 30 mg phosphatidylcholine dissolved in 1 ml of 2% dimethyl sulfoxide were diluted down 1 : 100 with 10% serum. The sarcolemmal preparation was incubated with this medium at 37 °C for 3 to 12 h under continuous of mild stirring. After this incubation period, the sacs were washed in the same way as explained above for the incorporated sarcolemmal sacs and the final pellets resuspended in a buffer containing 10 mM Tris-HCl, 2 mM dithiothreitol (pH 7.4). When [^{14}C]cholesterol (53 mCi/mmol) was employed, the same procedure for the incorporation and depletion of non-labelled cholesterol was used.

Measurement of the sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase activity. The sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase activity was assayed in an incubation medium containing 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 40 mM histidine, 120 mM Tris (pH 7.4). The incubation time utilized throughout the experiments using a temperature range of 40 °C to 0 °C was 10 min. The ATPase activities were determined every 3 °C between 0 °C and 25 °C and every 5 °C between 25 °C and 40 °C. In all instances, the reaction was initiated with the addition of ATP-Tris (pH 7.4) to give a final concentration of 2 mM and stopped with trichloroacetic acid to give a final concentration of 10%. All assays were carried out utilizing an average protein concentration of 100 to 200 μg of sarcolemmal sacs. The protein concentration of the different samples was assayed following the method described by Bradford [18] and the liberation of phosphate measured according to the method described by Fiske and SubbaRow [19].

Analysis of phospholipids. The phospholipids from the untreated sarcolemma and the membrane samples incorporated with cholesterol, were extracted according to Folch et al. [20]. Utilizing thin layer chromatography (Silica gel G plates 20 × 20 cm.) and a solvent system composed of $CHCl_3$/methanol (2 : 1, v/v), the phospholipids were separated in parallel with phospholipid standards. The plates were evaporated under nitrogen and according to the R_f of the different standards, the samples were scraped from the plates and dissolved in $CHCl_3$. The extracted phospholipids were hydrolyzed with concentrated perchloric acid at 150 °C for 30 min and centrifuged for 5 min at

3000 × g. After this procedure the phosphate concentration of the supernatants was measured following the method of Bartlett [21].

Fatty acid analysis. The sarcolemmal fatty acid composition was analyzed by gas-liquid chromatography. Ester moieties of the lipid fraction were transformed to their methyl esters following the method of Luddy et al. [22]. The samples were treated with 0.5 M NaOH/methanol for 15 min at 50 °C. After cooling at room temperature, the solution was treated with BF_3/methanol for 15 min at 50 °C and the methyl esters extracted with carbon disulfide. The methyl esters were injected into a gas chromatograph (Perkin-Elmer, Sigma model) having a column (2 m × 3 mm, i.d.) packed with 10% diethylene glycol succinate polyester and flame ionization detector. The flow rate of nitrogen was 30 ml/min and column temperature 170 °C. The fatty acid composition was analyzed by calculating the peak area of the samples to that of the internal standard. The fatty acid composition was expressed as a percentage of total fatty acids.

Results

During the following experiments, we attempted the study of the cholesterol effect upon the sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase by varying the cholesterol content of the sarcolemma.

In order to in vivo increase the cholesterol concentration of these membranes, the rabbits were fed with a high cholesterol diet and only when their plasma cholesterol level after 3 to 4 weeks had increased, the cardiac sarcolemmal fraction was isolated. The plasma cholesterol level from control animals was 62.25 ± 5.77 mg cholesterol/100 ml plasma (mean ± S.E. of 13 determinations from different animals) and increased to 209.57 ± 8.82 mg cholesterol in cholesterol-fed animals (mean ± S.E. of 39 determinations from different animals). When the cholesterol analysis was carried out using the sarcolemmal fraction obtained from the cholesterol fed rabbits, it was observed that the cholesterol concentration of these membranes) increased from 60.10 ± 2.09 to 127.96 ± 12.10 μg cholesterol/mg protein (mean ± S.E. of 13 different preparations).

When the incorporation experiment was carried out in vitro, the cholesterol concentration in the sarcolemma was also increased to 106.85 ± 9.41 μg cholesterol/mg protein (mean ± S.E. of six different preparations), opposite to the result observed during the depletion assay where the sterol concentration decrease to 30.18 ± 9.10 μg cholesterol (mean ± S.E. of six different preparations) below the normal value of 68.16 μg cholesterol/mg protein obtained for normally fed rabbits.

As shown in Fig. 1A, the Arrhenius activation energy (E_a) for the $(Ca^{2+} + Mg^{2+})$-ATPase activity from the control sarcolemmal sacs was increased as the temperature was lowered, showing a triphasic curve when the temperature range of 0 to 40 °C was employed. The transition temperatures were observed at 25 °C and between 7 °C and 10 °C for the low-temperature range. When the $(Ca^{2+} + Mg^{2+})$-ATPase activity of the sarcolemmal fractions isolated from cholesterol fed rabbits was measured and the logarithm of the activity plotted, despite the increase in the cholesterol level only a slight decrease in the ATPase activity was observed with no measurable changes in the transition temperatures (Fig. 1B). Although under these conditions there were slight changes in the E_a for the $(Ca^{2+} + Mg^{2+})$-ATPase, the differences between the two conditions were not at all pronounced (Fig. 1). Only when the sarcolemmal sacs were in vitro depleted of cholesterol, an important stimulation of the ATPase activity was observed (Fig. 2). However, despite the differences in the cholesterol content between depleted and in vitro incorporated sacs, the transition temperatures were also kept constant (Fig. 2).

In order to investigate if the changes in ATPase activity caused by the incorporation and depletion of cholesterol to the sarcolemma were reversible, the following set of experiments was designed. As shown in Fig. 3, it was observed that the sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase activity was inversely proportional to the cholesterol concentration of the membranes, showing a low ATPase activity with a high cholesterol content, and vice-versa, a high ATPase activity with a low cholesterol concentration. The consistent difference between the enzyme activity observed during this set of experiments and the ones discussed above, might be explained as a result of the utilization of different membrane batches for the different experimen-

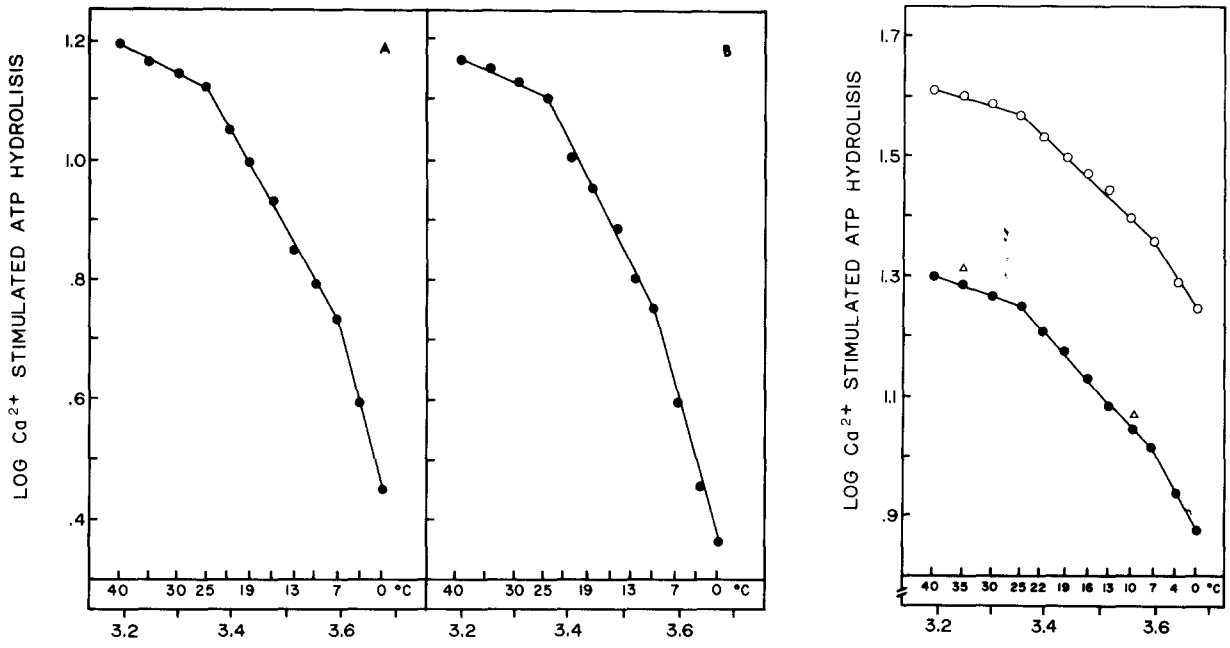

Fig. 1. Arrhenius plots of the sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol P_i/min per mg protein). (A) Sarcolemmal sacs isolated from normally fed rabbits; the Arrhenius activation energies (E_a) for the $(Ca^{2+} + Mg^{2+})$-ATPase activity from high to low temperatures correspond to 2.167, 8.049 and 14.306 kcal/mol. (B) Sarcolemmal sacs isolated from cholesterol fed rabbits; E_a for the $(Ca^{2+} + Mg^{2+})$-ATPase activity from high to low temperatures correspond to 1.665, 8.999 and 13.608 kcal/mol. Experimental details as explained in Materials and Methods (representative experiment).

Fig. 2. Arrhenius plots of the $(Ca^{2+} + Mg^{2+})$-ATPase activities (μmol P_i/min per mg protein) measured in sarcolemmal sacs depleted or incorporated with cholesterol in vitro. $(Ca^{2+} + Mg^{2+})$-ATPase activity from control sarcolemmal sacs measured before the depletion or the incorporation incubation (Δ). Activity measured in cholesterol-depleted membranes (O——O) and cholesterol incorporated membranes (●——●). The Arrhenius activation energies (E_a) for the depleted sarcolemmal sacs from high to low temperatures correspond to 1.521, 5.180 and 6.560 kcal/mol. The E_a for the incorporated sarcolemmal sacs correspond to 1.464, 4171 and 5.860 kcal/mol. Experimental details as explained in Materials and Methods (representative experiment).

tal designs.

Although it was shown in Fig. 3 that the changes in the ATPase activity due to variation in the membrane cholesterol concentration were totally reversible, after the depletion procedure despite the complete recovery of the ATPase activity, the cholesterol concentration did not go back to basal levels. This result might indicate a higher susceptibility of the enzyme to the withdrawal of cholesterol than to the incorporation of this sterol to the sarcolemma.

The use of labelled cholesterol showed that during the first minutes of cholesterol depletion, the drop in the cholesterol concentration was more important than the increase in the cholesterol level during the first minutes of incorporation of this sterol to the membranes (Fig. 4).

In order to verify if the stimulatory and inhibitory effects observed during this investigation were not due to a modification in the phospholipid content of the membranes, an analysis of the major sarcolemmal phospholipids was performed. Table I shows that despite the modification of the sarcolemmal cholesterol content observed when the lipoprotein system was used, the concentration of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol and cardiolipin remained stable, showing that there was no exchange of phospholipids between the plasma lipoproteins and the sarcolemmal sacs.

Furthermore, although the phospholipid/protein ratio remained constant, the modification of the enzyme activity could have been also due to changes in the fatty acid composition of the

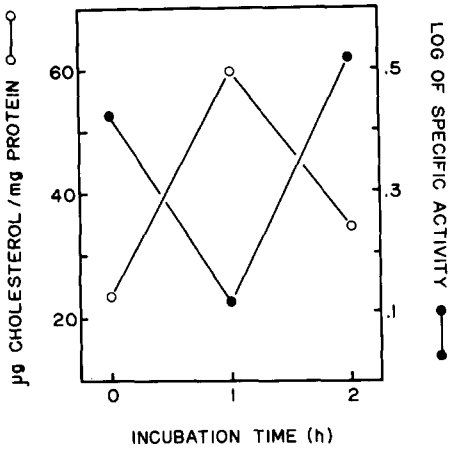

Fig. 3. Recovery of the $(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol P_i/min per mg protein) with depletion of the incorporated cholesterol from sarcolemmal sacs. The membranes were incubated for one hour in the incorporation medium before changing to the depletion one. Sarcolemmal cholesterol concentration (○). Sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase activity (●). Experimental details as explained in Materials and Methods (representative experiment).

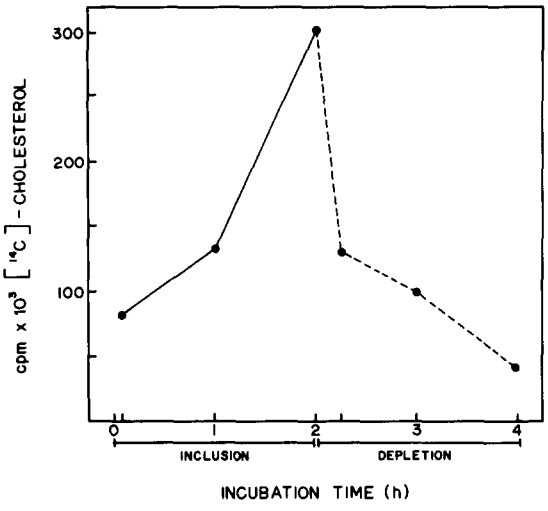

Fig. 4. Time-course of [^{14}C]cholesterol (53 mCi/mmol) inclusion and depletion of the incorporated sterol from sarcolemmal sacs. The membranes were incubated for 2 h in the incorporation medium before changing to the depletion one. Experimental details as explained in Materials and Methods (representative experiment).

sarcolemma, these residues were determined in control sarcolemmal sacs and cholesterol incorporated sarcolemma. Table II shows that despite the treatment for the incorporation of cholesterol to these membranes, the concentration of fatty acid residues remains stable as in untreated sarcolemma. These results suggest that the changes in $(Ca^{2+} + Mg^{2+})$-ATPase activity observed after the modification of the membrane cholesterol level are not due to an alteration of the phospholipid or fatty acyl composition of the sarcolemma.

TABLE I

SARCOLEMMAL PHOSPHOLIPID CONTENT

Figures are presented as means ± S.E. of seven different preparations.

	Phospholipids (nmol/mg protein)	
	Control sarcolemma	Cholesterol incorporated sarcolemma
Phosphatidylinositol	70.52 ± 10.79	71.59 ± 5.71
Phosphatidylethanolamine	80.41 ± 13.14	79.39 ± 9.36
Phosphatidylcholine	129.18 ± 20.06	97.39 ± 17.10
Phosphatidylserine	75.82 ± 8.20	76.89 ± 10.10
Cardiolipin	63.15 ± 5.62	63.21 ± 3.78

Discussion

The experimental results presented in this study indicate that cholesterol modulates the $(Ca^{2+} + Mg^{2+})$-ATPase activity from cardiac sarcolemma as equally shown for other membrane-located

TABLE II

FATTY ACID COMPOSITION OF CARDIAC MUSCLE SARCOLEMMA

Values representative of three experiments are expressed as the percentage of total fatty acids. NI, non identified.

	% of total fatty acids	
	Control sarcolemma	Cholesterol incorporated sarcolemma
NI	0.57	0.44
16:0	26.17	26.61
NI	1.96	1.43
18:0	12.82	12.38
18:1	17.35	17.67
18:2	25.50	26.00
18:3	0.51	0.81
20:0	1.86	1.63
20:4	13.26	13.02

ATPases [12–14]. However, while these reports show that cholesterol mainly produces an inhibitory effect when incorporated into membranes, our results seem to point out to the fact that in our system although an inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity is seen after the incorporation of cholesterol to the sarcolemma, depletion of this sterol induces a much more pronounced affect observed as an increase in the $(Ca^{2+} + Mg^{2+})$-ATPase activity. This observation might indicate that the sarcolemma contains a critical concentration of cholesterol that normally modulates the ATPase activity. When the concentration of this sterol is several times increased, although an inhibition of the ATPase activity is observed, the inhibition seems not to be proportional to the amount of cholesterol incorporated. In contrast, when a small amount of cholesterol is withdrawn from the sarcolemmal sacs, an important stimulation of the ATPase activity is observed. Since it could be suggested from the slight changes in E_a and transition temperatures that either an incorporation or a depletion of cholesterol from the sarcolemmal sacs in our system did not have an important effect upon the fluidity of the membranes, a possible explanation for the changes in activity caused by changing the sarcolemmal cholesterol concentration could have been given as a direct effect of cholesterol upon the sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase. Although it has been suggested that the boundary lipid might protect the enzyme activity from changing with variations in the lipid composition of the membrane [23,24], our results indicate the possibility of a slow exchange of cholesterol between the tightly bound lipid surrounding the $(Ca^{2+}Mg^{2+})$-ATPase and the bulk lipid of the sarcolemma.

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